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Original Paper

Chlamydia psittaci is variably associated with ocular adnexal MALT lymphoma in different geographical regions

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Abstract

Infectious agents play a critical role in MALT lymphoma development. Studies from Italy showed *Chlamydia psittaci* infection in 87% of ocular adnexal MALT lymphomas and complete or partial regression of the lymphoma after *C. psittaci* eradication in four of nine cases. However, *C. psittaci* was not demonstrated in ocular adnexal MALT lymphomas from the USA. This study was thus designed to investigate further the role of *C. psittaci*, and other infectious agents commonly associated with chronic eye disease, in the development of ocular adnexal MALT lymphoma. The presence of *C. psittaci*, *C. trachomatis*, *C. pneumoniae*, herpes simplex virus 1 and 2 (HSV1, HSV2), and adenovirus 8 and 19 (ADV8, ADV19) was assessed separately by polymerase chain reaction in 142 ocular adnexal MALT lymphomas, 53 non-marginal zone lymphomas, and 51 ocular adnexal biopsies without a lymphoproliferative disorder (LPD), from six geographical regions. *C. psittaci* was detected at similar low frequencies in non-LPD and non-marginal zone lymphoma groups from different geographical regions (0–14%). Overall, the prevalence of *C. psittaci* was significantly higher in MALT lymphomas (22%) than in non-LPD (10%, $p = 0.042$) and non-marginal zone lymphoma cases (9%, $p = 0.033$). However, the prevalence of *C. psittaci* infection in MALT lymphoma showed marked variation among the six geographical regions examined, being most frequent in Germany (47%), followed by the East Coast of the USA (35%) and the Netherlands (29%), but relatively low in Italy (13%), the UK (12%), and Southern China (11%). No significant differences in the detection of *C. pneumoniae*, *C. trachomatis*, HSV1, HSV2, ADV8, and ADV19 were found between lymphomas and controls from different geographical regions. In conclusion, our results show that *C. psittaci*, but not *C. pneumoniae*, *C. trachomatis*, HSV1, HSV2, ADV8 or ADV19, is associated with ocular adnexal MALT lymphoma and that this association is variable in different geographical areas.

Keywords: ***Chlamydia psittaci*; ocular adnexal MALT lymphoma**

Introduction

Extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) arises at a number of extranodal sites including the gastrointestinal tract, salivary and thyroid glands, lung, ocular adnexa, and skin. Interestingly, these organs are devoid of native lymphoid tissue: lymphoma at these sites arises from the MALT acquired as a result of a chronic inflammatory or autoimmune disorder [1]. The inflammatory disease associated with MALT lymphoma not only provides a microenvironment that is

crucial for malignant transformation, but the immunological response generated during the inflammatory process also promotes the growth of the lymphoma cells. This is best exemplified in gastric MALT lymphoma, which is driven by *Helicobacter pylori* mediated immune responses and can be effectively treated by eradication of the bacterium in the majority of cases [2–4]. Similarly, *Borrelia burgdorferi* and *Campylobacter jejuni* infections are associated with cutaneous marginal zone B-cell lymphoma and immunoproliferative small intestinal disease respectively, and eradication of these organisms resulted in complete regression of the lymphoma in some cases [5–10]. Together, these findings suggest that the development of MALT lymphomas at other sites may also be associated with infectious agents.

Ocular adnexal MALT lymphoma represents a significant proportion (approximately 12%) of all MALT lymphomas [11] and is the most common lymphoma of the ocular adnexa [12–14], occurring principally in the conjunctiva, orbital soft tissue, and lachrymal apparatus. Analogous to the evolution of gastric MALT lymphoma from *H. pylori* associated chronic gastritis, ocular adnexal MALT lymphoma may be associated with chronic conjunctivitis; interestingly, there is a considerable overlap in both the histological and clinical presentations of chronic conjunctivitis and ocular adnexal MALT lymphoma [15–17]. Ocular adnexal MALT lymphoma thus may arise from the MALT acquired as a result of chronic inflammatory responses. Infectious agents underlying chronic eye infection, particularly those involved in chronic conjunctivitis such as *Chlamydia*, herpes simplex virus (HSV), and adenovirus (ADV) [18–22], may therefore play a role in the development of lymphoma.

The aetiology of ocular adnexal MALT lymphoma is currently unclear. Recent studies from Italy showed evidence of *Chlamydia psittaci* (*C. psittaci*) infection in 87% of ocular adnexal MALT lymphomas [23], and eradication of the organism by antibiotics led to complete or partial regression of the disease in four of nine cases studied [24]. However, such an association was not demonstrated in cases of ocular adnexal MALT lymphoma from South Florida and Rochester (New York) areas in the USA [25,26]. This raises the possibility that *C. psittaci* may be variably associated with ocular adnexal MALT lymphoma in different geographical regions and that other aetiological factors may be involved in the development of this lymphoma. To examine these issues, we screened for infectious agents underlying chronic eye infection, namely *C. psittaci*, *C. trachomatis*, *C. pneumoniae*, HSV types 1 and 2, and ADV types 8 and 19, in ocular adnexal lymphomas of various subtypes as well as ocular adnexal biopsies without a lymphoproliferative disorder (LPD), from six geographical regions.

Materials and methods

Tissue specimens

Archival formalin-fixed paraffin-embedded ocular adnexal biopsies from 263 patients from six geographical areas, obtained between 1981 and 2005, were analysed. Of these cases, 246 had adequate material as judged by quality control polymerase chain reaction (PCR; detailed in a later section), and were thus suitable for PCR screening of infectious agents. These included a total of 195 lymphomas, consisting of 142 MALT lymphomas and 53 non-marginal

Table 1. Demographic and histological characteristics of ocular adnexal lymphomas and controls from different geographical areas

Characteristics	UK[1]	Germany	Netherlands	Italy[2]	Southern China[3]	East coast USA[4]	Total
No of patients	80	37	24	21	59	25	246
Median age (range)	64 (17–93)*	58 (8–95)	62 (19–93)	61 (37–86)	60 (32–87)‡	69 (28–90)	62 (8–95)
Male/female ratio	0.9*	1.3	0.6	0.5	2.6‡	1.3	1.3
Diagnosis							
MALT L	33	19	21	15	37	17	142
Non-MZL[5]	7	9	3	6	20	8	53
Non-LPD[6]	40	9	—	—	2	—	51
Anatomical localization							
Conjunctiva	50†	22	22	9	1	10	114
Orbit	5†	13	2	10	49	9	88
Other[7]	6†	2	—	2	9	6	25

[1] 51 cases from London, 19 cases from Manchester, and 10 cases from Cambridge.
[2] 13 cases from Ancona and eight cases from Bologna.
[3] 29 cases from Canton, 15 cases from Hainan, and 15 cases from Shanghai.
[4] 18 cases from northeast, three from mid-east, and four from southeast coast.
[5] 21 follicular lymphomas, 13 mantle-cell lymphomas, 11 diffuse large B-cell lymphomas, and eight T/NK-cell lymphomas.
[6] 39 cases were conjunctival biopsies from unselected autopsies with no prior history of conjunctival or ocular disease [27] and the remaining cases were mainly pinguecula and occasionally chalazion.
[7] Eyelid, lachrymal gland, extraocular muscle, globe, and three ocular adnexal cases without details of biopsy site.
* Excluding 19 cases from Manchester and non-LPD cases, for which data are not available.
† Excluding 19 cases from Manchester, for which data are not available.
‡ Excluding 29 cases from Canton, for which data are not available.
No = number; LPD = lymphoproliferative disorder; MZL = marginal zone lymphoma; MALT L = mucosa-associated lymphoid tissue lymphoma.

zone lymphomas, as well as 51 ocular adnexal biopsies without any histological evidence of a LPD (Table 1). Cases were diagnosed or reviewed by haematopathologists. Table 1 summarises the anatomical location of these biopsies together with the patients' age and sex. Local ethical guidelines were followed for the use of archival paraffin embedded tissues for research, and such use was approved by the local ethics committees of the authors' institutions where required.

DNA extraction

Tissue sections (3–5 μm) were dewaxed in xylene and washed in ethanol. DNA was extracted and purified using QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions, and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Quality control PCR

The quality of each DNA sample was assessed by PCR amplification of variously sized human gene fragments (100 bp, 200 bp, 300 bp, and 400 bp) (Table 2) [28]. A multiplex PCR was carried out using ABgene Thermo-Start DNA polymerase (Surrey, UK) following the supplier's protocol. PCR products were analysed by electrophoresis on 6% polyacrylamide gels. Only cases with successful amplification of a 200 bp or larger product were used to screen for infectious agents

Detection of infectious agents by PCR

Stringent laboratory procedures for PCR set-up and product analyses were carefully followed to avoid any potential cross contamination. PCRs without template DNA were randomly interspersed among test samples to monitor potential cross contamination. Procedures for the detection of each *Chlamydia* species were validated by a double blind comparison of two series of DNA samples with known chlamydial status between our laboratory and Dr Dolcetti's laboratory.

Chlamydiae

The detection of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* was carried out using a previously described Touchdown Enzyme Time-Release (TETR) PCR [23,29], with the following modifications. Instead of multiplex PCR, separate PCRs for each *Chlamydia* species were performed. For *C. trachomatis* and *C. pneumoniae*, new primer sets were designed to target smaller fragments of the 16S rRNA gene (Table 2), thus suitable for screening DNA samples prepared from paraffin-embedded tissues. Additionally, higher touchdown annealing temperatures from 66 °C to 56 °C were used for *C. trachomatis* detection. For all *Chlamydiae* screening, PCR amplifications were carried out in a 25 μl reaction mixture containing 150 ng of template DNA. PCR products were analysed by electrophoresis on 10% polyacrylamide gels.

In each case, three independent PCR amplifications were carried out for each *Chlamydia* species. To make data comparable, we adopted the approach by Ferreri *et al* [23] and only cases with positive PCR results in at least two of the three independent reactions were regarded as true positives.

Adenovirus and Herpes simplex virus

ADV types 8 and 19 were separately detected by PCR amplification of the viral hexon gene (Table 2). ADV8 PCR was carried out in a 25 μl reaction mixture

Table 2. PCR primers used for DNA quality assessment and the molecular detection of infectious agents

	Gene	Primer name	Sequence	Product size (bp)
Quality control [28]	TBXAS1 exon9	Q 100s	5′ GCCCGACATTCTGCAAGTCC 3′	100
		Q 100as	5′ GGTGTTGCCGGGAAGGGTT 3′	
	Recombination activating gene 1 (RAG1) exon2	Q 200s	5′ TGTTGACTCGATCCACCCCA 3′	200
		Q 200as	5′ TGAGCTGCAAGTTTGGCTGAA 3′	
	Promyelocytic leukaemia zinc finger (PLZF) exon1	Q 300s	5′ TGCGATGTGGTCATCATGGTG 3′	300
		Q 300as	5′ CGTGTCATTGTCGTCTGAGGC 3′	
	ALL1 fused gene from chromosome 4 (AF4) exon11	Q 400s	5′ CCGCAGCAAGCAACGAACC 3′	400
		Q 400as	5′ GCTTTCCTCTGGCGGCTCC 3′	
C. psittaci [29]	16S rRNA and 16S–23S spacer rRNA	CPS 100s	5′ CCCAAGGTGAGGCTGATGAC 3′	111
		CPS 101as	5′ CAAACCGTCCTAAGACAGTTA 3′	
C. pneumoniae [29]	16S rRNA	CPN 73s	5′ ATTCGATGCAACGCGAAGGACCT 3′	73
		CPN 91as	5′ TGCGGAAAGCTGTATTTCTACAGTT 3′	
C. trachomatis	16S rRNA	CTR 116s	5′ TATTTGGGCATCCGAGTAACG 3′	116
		CTR 116as	5′ AGACGTCATAGCCTTGGTAGGCC 3′	
Adenovirus type 19	Hexon gene for major capsid protein	ADV19 87s	5′ AACCAGCCAAAGAGGATGAAAG 3′	87
		ADV19 87as	5′ TGTCTCTGAAGCCAATGTAGTTG 3′	
Adenovirus type 8	Hexon gene for major capsid protein	ADV8 65s	5′ ATGTGGAACTCTGCGGTGGACA 3′	65
		ADV8 65as	5′ TCCACACCGTGATTCTCAAT 3′	
Herpes simplex virus type 1 and 2 [30]	DNA polymerase gene	HSV1/2 92s	5′ CATCACCGACCCGGAGAGGGAC 3′	92
		HSV1/2 92as	5′ GGGCCAGGCGCTTGTTGGTGTA 3′	

using ABgene Thermo-Start DNA polymerase. The touchdown protocol used consisted of 95 °C × 30 s, 63 °C × 45 s (decreased 1 °C every two cycles until 60 °C), and 72 °C × 30 s, followed by 35 cycles with the annealing temperature at 59 °C. The cycle parameters for ADV19 PCR were identical to those used for *C. trachomatis* PCR.

HSV types 1 and 2 (HSV1/2) were simultaneously screened with a common primer set [30] (Table 2). The cycle parameters for HSV1/2 PCR were identical to those used for ADV8 PCR.

PCR products were analysed by electrophoresis on 10% polyacrylamide gels. As with our assays for *Chlamydiae*, a case was considered positive when the virus was detected in at least two of three PCRs.

DNA sequencing

PCR products from selected cases of different geographical origins were purified and sequenced in both orientations using an ABI 377 DNA sequencer (ABI PRISM Perkin Elmer Warrington, UK). Sequences were analysed by BLAST search of the NCBI database (http://www.ncbi.
nlm.nih.gov/blast, accessed 9 March 2006).

Statistical analysis

Differences in the prevalence of infectious agents among various lymphoma subtypes and controls were analysed using Fisher's exact test ("stats Package" in R version 2.1.1).

Results

C. psittaci is detected at variable frequencies in ocular adnexal MALT lymphomas from different geographical regions

A total of 246 ocular adnexal biopsies were assessed for the presence of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* by separate PCRs (Figure 1). Quality control did not show any differences in the quality of DNA samples from lymphoma specimens from the various geographical regions. We sequenced 17 *C. psittaci*, 10 *C. trachomatis*, and 10 *C. pneumoniae* PCR products from different geographical regions and all were confirmed to be specific, demonstrating the reliability of the method. With the exception of one *C. psittaci* and two *C. pneumoniae* PCR products, each of which contained a single nucleotide change, the PCR products sequenced did not show any sequence variations.

Overall, 31/142 (22%) cases of MALT lymphoma from all regions were positive for *C. psittaci*, a frequency significantly higher than that observed in both non-LPD samples ($p = 0.042$) and non-marginal zone lymphoma samples ($p = 0.033$) from all areas. *C. psittaci* DNA was detected at broadly similar frequencies in both non-LPD and non-marginal zone lymphoma groups from different geographical regions (0–14%) (Table 3). In contrast, the bacterium was found at variable prevalences in MALT lymphomas from different regions, being more frequent in Germany (47%), followed by the East Coast of the USA (35%) and the Netherlands (29%), but relatively low in

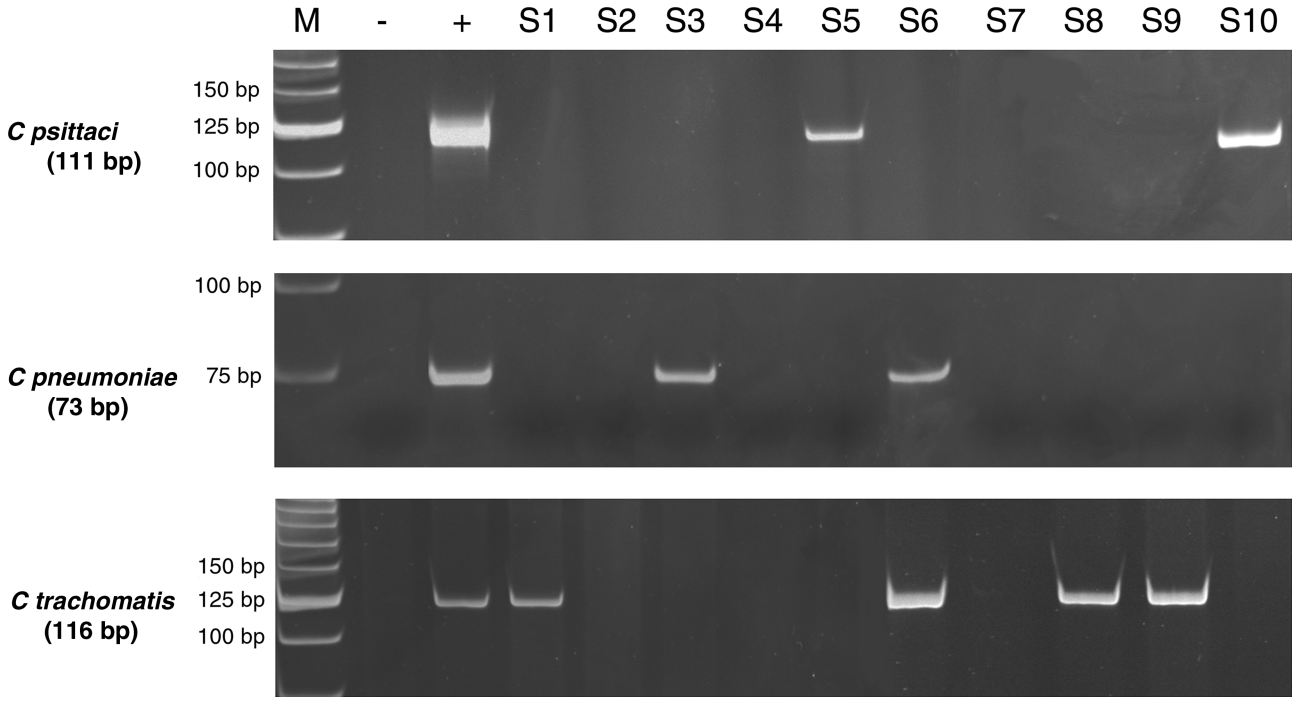

Figure 1. PCR detection of *C. psittaci*, *C. pneumoniae*, and *C. trachomatis* DNA in ocular adnexal MALT lymphoma specimens (10% polyacrylamide gel). M, molecular weight marker; −/+, negative/positive controls. S1–S10: MALT lymphomas (S1–S3 Würzburg, Germany; S4–S5 Manchester, UK; S6–S7, Bologna, Italy; S8, Hainan, China; S9–S10, Canton, China). S6: positive for both *C. pneumoniae* and *C. trachomatis*

Table 3. Frequencies of *Chlamydiae* detected in ocular adnexal lymphomas and controls from different geographical areas*

		UK		Germany		Netherlands		Italy		Southern China		East coast USA		Total	
Chlamydiae	**Diagnosis**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
C. psittaci	MALT L	4/33	12	9/19	47[1]	6/21	29	2/15	13	4/37	11	6/17	35[2]	31/142	22[3]
	Non-MZL†	1/7	14	0/9	0	2/3		0/6	0	2/20	10	0/8	0	5/53	9
	Non-LPD	4/40	10	1/9	11	—	—	—	—	0/2		—	—	5/51	10
C. pneumoniae	MALT L	5/30	17	4/19	21	0/12	0	0/15	0	8/37	22	0/14	0	17/127	13
	Non-MZL†	0/5		0/9	0	0/3		1/6	17	2/20	10	0/4		3/47	6
	Non-LPD	10/40	25	2/9	22	—	—	—	—	0/2		—	—	12/51	24
C. trachomatis	MALT L	6/30	20	1/19	5	2/12	17	1/15	7	0/37	0	2/14	14	12/127	9
	Non-MZL†	3/5		0/9	0	1/3		0/6	0	0/20	0	1/4		5/47	11
	Non-LPD	10/40	25	0/9	0	—	—	—	—	0/2		—	—	10/51	20

* A case was regarded as positive if the screened bacterium was detected in at least two of three independent PCRs [23]. Percentages are only provided for groups with more than five cases.

† Follicular lymphoma, mantle-cell lymphoma, diffuse large B-cell lymphoma, and T/NK-cell lymphoma.

[1] Significantly different from non-MZLs from Germany ($p = 0.013$) and also significantly different from MALT lymphomas from the UK ($p = 0.007$), Italy ($p = 0.039$), and Southern China ($p = 0.004$).

[2] Significantly different from MALT lymphomas from Southern China ($p = 0.041$).

[3] Significantly different from all non-LPDs ($p = 0.042$) and from all non-MZLs ($p = 0.033$).

MALT L = mucosa-associated lymphoid tissue lymphoma; MZL = marginal zone lymphoma; LPD = lymphoproliferative disorder.

Italy (13%), the UK (12%), and Southern China (11%) (Table 3). Interestingly, three of the six positive cases detected within our USA group were from Florida and New York, where no association was found between *C. psittaci* infection and ocular adnexal MALT lymphomas in previous studies [25,26]. The prevalence of *C. psittaci* in MALT lymphomas from Germany was significantly higher than those observed in the UK, Italy, and Southern China ($p < 0.04$). There was no correlation between *C. psittaci* positivity and either the age or sex of the patients or the anatomical location of the lymphoma. The prevalence of *C. psittaci* positivity was nearly identical between cases that occurred in the orbit (15%) and those involving the conjunctiva (16%). In addition, there was no apparent difference in the prevalence of *C. psittaci* among various subtypes of non-marginal zone lymphomas.

A total of 225 cases with sufficient DNA quantity were screened for the presence of *C. pneumoniae* and *C. trachomatis*. Both bacteria were found at variable frequencies in non-LPD and non-marginal zone lymphoma groups from different geographical regions (0–25%) (Table 3). Only cases from Southern China showed a trend towards a higher prevalence of *C. pneumoniae* in MALT lymphomas than in non-LPD and non-marginal zone lymphoma cases from the same region (Table 3), but these differences were not statistically significant. There was no difference in the prevalences of *C. trachomatis* between MALT lymphoma and control groups, both non-LPDs, and non-marginal zone lymphomas, from the same geographical regions. In general, the presence of these *Chlamydiae* was mutually exclusive.

To examine whether there was any correlation between the positivity for *Chlamydiae* and the extent of lymphoid infiltration in the non-LPD group, the histology of these cases was reviewed. These specimens typically showed variable infiltrates of mature lymphocytes and plasma cells within the lamina propria, and some contained a few intraepithelial neutrophils. Occasionally, the infiltrating lymphocytes formed small aggregates but no lymphoid follicles were seen. There was no correlation between the positivity for *Chlamydiae* and the extent of lymphoid infiltration in these control samples.

Adenovirus and herpes simplex virus are not associated with ocular adnexal lymphoma

A total of 152 cases with adequate DNA quality had a sufficient quantity of DNA to screen for the presence of ADV8, ADV19 and HSV1/2. Sequencing confirmed the specificity of the PCR products in all positive cases. Only low levels of positivity (0–14%) for these viruses were found in both control and MALT lymphoma groups. No difference was seen in the prevalences of these viruses between MALT lymphoma and control groups from different geographical regions.

Discussion

By retrospective investigation of archival ocular adnexal MALT lymphomas from six geographical regions, we provide evidence that *C. psittaci* is associated with ocular adnexal MALT lymphoma but that this association is highly variable according to the geographical origin, with a frequency of *C. psittaci* in ocular adnexal MALT lymphoma ranging from 11% to nearly 50% in the different geographical areas examined. Importantly, these findings provide an explanation for the discrepancy between the original study by Ferreri *et al* (Milan, Italy) and subsequent reports: in Italy, *C. psittaci* was detected in 87% of ocular adnexal MALT lymphoma [23], while no evidence of

infection by this bacterium was demonstrated in cases from the South Florida and Rochester (New York) areas of the USA [25,26]. Such geographical variations are further supported by recent meeting abstracts showing a high prevalence of *C. psittaci* in ocular adnexal MALT lymphomas from South Korea (26/33) [31], but an absence or a low prevalence in cases from North America (0/15) [32] and Cuba (1/21) [33]. Interestingly, such a geographically variable association may also exist within the same country as shown by the differences in the prevalence of *C. psittaci* in ocular adnexal MALT lymphomas from Italy and the USA observed between the current study and previous reports [23,25,26,32].

The reasons underlying the marked variations in the prevalences of *C. psittaci* in ocular adnexal MALT lymphoma from different geographical regions are currently unknown. However, such geographical differences in linking infectious organisms with lymphoma, including within the same country, are not unprecedented, as the established associations between lymphoma and respectively hepatitis C virus, *Borrelia burgdorferi* and *H. pylori* are subject to marked geographical variations [34–36]. The prevalences of *C. psittaci* infection among the general populations of the geographical regions studied may vary, but epidemiological data specifically on ocular infection by *C. psittaci* are lacking. Given that the prevalence of *C. psittaci* infection in ocular adnexal MALT lymphoma is relatively low, at least in several geographical regions, and that variable prevalences are also found within the same country, the bacterial infection could be sporadic. Indeed, there is substantial evidence that *C. psittaci* infection in man is significantly associated with the environmental context, especially with exposure to pet birds and pet cats [37]. Interestingly, in the study by Ferreri [23], 13/24 interviewed patients with *C. psittaci*-positive ocular adnexal lymphomas had prolonged contact with household animals.

Overall, *C. psittaci* was found at a significantly higher prevalence in MALT lymphomas than in non-marginal zone lymphomas. The frequency of *C. psittaci* in non-marginal zone lymphoma cases was similar to the frequency observed in the non-LPD cases. These results suggest that the bacterium might be preferentially associated with MALT lymphoma, further implicating its role in the development of MALT lymphoma. In this context, it would be interesting to examine whether different strains of *C. psittaci*, with potentially different pathogenic capacities, could be differentially associated with various types of lymphoma, or with different geographical regions showing variable prevalences of *C. psittaci*-positive MALT lymphoma. Indeed, various strains of *H. pylori* have been shown to be differentially involved in several gastric diseases, with the virulent strains being preferentially associated with gastric cancer or peptic ulcer rather than with gastritis [38–40].

The demonstration of variable association of *C. psittaci* with ocular adnexal MALT lymphoma in different geographical regions is clinically important. In Italy, eradication of *C. psittaci* by antibiotics led to complete or partial regression of the disease in four of nine cases studied [24]. Patients who responded to antibiotics included some who did not respond to radiotherapy or chemotherapy, or showed repeated relapses of the disease after such treatment. Given that antibiotic treatment has relatively few side effects and ocular adnexal MALT lymphoma is an indolent disease, further clinical trials are warranted to determine the role of antibiotics in the treatment of this lymphoma. In this context, the prevalence of *C. psittaci* in ocular adnexal MALT lymphoma in a given geographical area is likely to be a major determinant of the value of such treatment in each clinical setting.

Among the six geographical regions examined in the current study, the highest prevalence of *C. psittaci* observed was in ocular adnexal MALT lymphomas from Germany (47%). This is much lower than the frequency observed in the original study from Italy (87%) [23]. It could be argued that the concentration of *C. psittaci* DNA in some of the tumour specimens might be low, potentially leading to underestimation of its true prevalence. However, the lower detection rate in the current study is unlikely to be due to a lack of sensitivity of our assays. Firstly, an identical PCR amplification of the same gene fragment specific to *C. psittaci* was used in both the original study and our current study. Secondly, we performed separate PCRs for each *Chlamydia* species and analysed PCR products on high-resolution gels, further ensuring the sensitivity and specificity of the method. The reliability of our data is supported by the finding that, in 80% of the positive cases, *C. psittaci* was detected in all three PCRs independently performed for each case.

Although we provide compelling evidence that ocular adnexal MALT lymphoma may develop from the MALT acquired as a result of chronic inflammation associated with *C. psittaci*, the low prevalence of *C. psittaci* in ocular adnexal MALT lymphoma in several geographical regions raises the possibility that other aetiological factors may be involved in the development of this lymphoma. Several other organisms, including *C. trachomatis*, *C. pneumoniae*, ADV types 8 and 19, and HSV types 1 and 2, are known to cause chronic eye infection [18–20]. We screened our cases for these infectious agents and found that their prevalences in both ocular adnexal MALT lymphoma and control groups were relatively low. Our results thus suggest that these infectious agents are unlikely to be associated with ocular adnexal MALT lymphoma, at least in the geographical regions investigated. Other aetiological factors underlying the development of adnexal MALT lymphoma remain to be investigated. Interestingly, some autoimmune disorders are known to be associated with an increased risk of lymphoma development [41,42], including MALT lymphoma [43–45], and some of them, such as systemic lupus erythematosus and Sjögren's syndrome,

often affect the ocular adnexa [46,47]. In this regard, it would be interesting to examine any possible role for autoimmunity in the development of ocular adnexal MALT lymphoma [48].

In summary, our results demonstrate that *C. psittaci* is variably associated with ocular adnexal MALT lymphoma in different geographical regions. Among different subtypes of ocular adnexal lymphomas, the bacterium appeared to be preferentially associated with MALT lymphomas. These findings have important clinical implications when considering the use of antibiotics to treat ocular adnexal MALT lymphomas.

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